

EVIDENCE FOR THE PRESENCE OF A GERMINATION INHIBITOR IN SEEDS OF *STRELITZIA* AIT.

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ABSTRACT

Although seeds of *Strelitzia* exhibit a condition of dormancy, excised embryos were found to be capable of prompt and normal growth when placed in a suitable medium. Growth was, however, markedly inhibited on addition of seed coats. This inhibitory effect was unaffected by the presence or absence of light or by five years of dry storage of the seed but was significantly reduced in seed coats of germinated seeds.

Using a lettuce seed germination biotest as well as a biotest involving excised *Strelitzia* embryos, chromatograms of aqueous seed extracts were found to contain inhibitory fractions.

UITTREKSEL

GETUIENIS VIR DIE TEENWOORDIGHEID VAN 'N ONTKIEMINGSINHIBEER-
DER IN SADE VAN *STRELITZIA* AIT.

Hoewel sade van *Strelitzia* in 'n rustoestand verkeer, besit geïsoleerde embrio's die vermoë om vinnig en normaal te groei wanneer hulle in 'n geskikte medium geplaas word. Byvoeging van die omringende weefsels inhibeer hierdie groei egter aansienlik. Hierdie inhiberende effek word nie deur die aan- of afwesigheid van lig beïnvloed nie, is nog teenwoordig na vyf jaar van droë opberging van die saad, maar toon 'n betekenisvolle afname in ontkiemende sade.

Deur gebruik te maak van 'n slaaisaad biotoets asook 'n toets met geïsoleerde *Strelitzia* embrio's is vasgestel dat chromatogramme van waterige ekstrakte van die saad inhiberende fraksies bevat.

INTRODUCTION

Although seeds of the acaulescent species of *Strelitzia* do not germinate promptly, normal and rapid growth of excised embryos takes place when they are incubated in a suitable medium (Van de Venter & Small, 1974). This would seem to indicate that some or other factor, residing in the seed coat*, is responsible for dormancy.

Seed coats may be responsible for dormancy in various ways (Wareing, 1969; Egle, 1972). One possibility is that dormancy is maintained by means of germination inhibitors contained in the seed coat. Evidence that this mechanism could be operative in the case of *Strelitzia* is presented in this paper.

*The term *seed coat* as used in this paper denotes all the tissues in the seed surrounding the embryo.

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MATERIALS AND METHODS

Embryo cultures

Excised embryos of *S. juncea* were used throughout as greater uniformity in growth was obtained with this material as was the case with excised embryos of *S. reginae*. No morphological differences between either the seeds or embryos of these species is apparent. In fact, the only obvious difference between these two species is a distinct difference in leaf morphology (Van de Venter, Small and Robbertse, 1975). To prepare the seeds for removal of their embryos, they were abraded and surface-sterilized by immersion for 2 minutes in concentrated sulphuric acid, rinsing for 1 minute in fast flowing tap water, and then immersion for 30 minutes in a 0.2% solution of mercuric chloride (HgCl_2). The seeds were then rinsed with one litre of sterile, distilled water and placed overnight on moist filter paper in sterile petri dishes. This imbibition period softened the seed coats sufficiently for the embryos to be removed with comparative ease.

The seed coats of these seeds were discarded and the embryos subsequently cultured in sterile White's nutrient solution (White, 1963) without vitamins. Two embryos were incubated per 30 ml of culture solution contained in conical flasks of 100 ml capacity.

Where seed coats were also added to the culture solutions, these were obtained from seeds of *S. juncea* or *S. reginae*, depending on the particular experiment. Whole, unabraded seeds were surface-sterilized in H_2SO_4 and HgCl_2 as described above and then rinsed with three, one litre volumes of sterile, distilled water. These seeds were not abraded because of the possibility that the exposed endosperm could absorb mercuric chloride. Water uptake by these seeds was slower than in the case of abraded seeds and they were therefore allowed to imbibe for three days as a softening procedure. The seeds were then cut in half and the embryos discarded. The seed coats of two seeds were added to the solution in each flask.

The flasks containing the embryos were continuously agitated on an orbital shaker, in the dark, at $25 \pm 0.5^\circ \text{C}$ in a Controlled Environments E7H growth cabinet. After twelve days of incubation, growth was determined in terms of root length. The data were subjected to statistical analyses and lowest significant differences computed according to the method of Tukey (Steel and Torrie, 1960).

Extraction and chromatography

Twenty-five grams of month-old seeds of *S. reginae* were imbibed in distilled water for six days. The seeds were then frozen in liquid nitrogen, ground in a coffee mill and extracted three times (24 hour periods) with 200 ml volumes

of distilled water. The extraction was performed at 4 °C under nitrogen. The extracts were pooled and filtered through a disc of Whatman number 42 filter paper after which the filtrate was concentrated by freeze-drying.

The concentrate was taken up in a little water and streaked onto Whatman number one chromatography paper. The chromatogram was developed by the descending method using isopropanol: 25% ammonia: water (10:1:1) as solvent. After the solvent front had moved 30 cm the chromatograms were dried at 35 °C for ten minutes. The chromatograms were cut into ten equal, transverse sections and the biological activity of each section was determined.

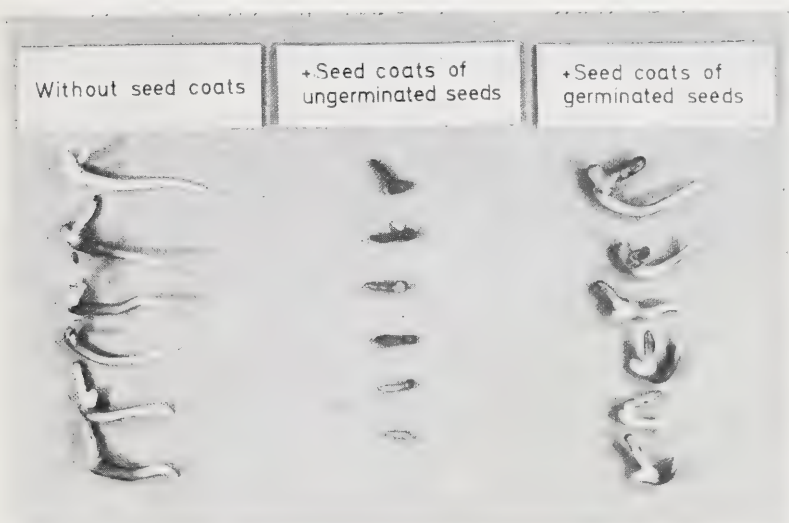


FIG. 1.

Effect of seed coats from germinated and ungerminated *S. reginae* seeds on the growth of *S. juncea* embryos.

Bioassays

A lettuce seed germination test similar to that used by Black and Wareing (1959) was employed using the non-light requiring cultivar "Great Lakes". The seeds were incubated at 25 ± 0.5 °C and germination counts made after 48 hours.

It was realised that, although this bioassay was specific for germination inhibitors, more conclusive results would be obtained by making use of *Strelitzia* material. Adding chromatogram sections to excised *S. juncea* embryos in

White's medium and measuring growth after twelve days did not give satisfactory results. No satisfactory method for the sterilization of chromatogram sections could be found and contamination of the growth medium by micro-organisms was consequently severe. The variation in growth between different embryos also proved to be a major problem.

A biotest was therefore devised in which embryos were allowed to grow for nine days before the addition of chromatogram sections. Growth was measured on the twelfth day, that is, on the third day after addition of chromatogram sections. No contamination by micro-organisms was visible by the twelfth day as the period of incubation was probably too short to allow a large-scale multiplication of microbes. Variation in embryo growth was reduced to a minimum in this biotest by cultivating an abundance of embryos and visually selecting for uniformity on the ninth day.

RESULTS

Experiments with excised embryos

In the first experiment the effect of adding seed coats of four-month-old seeds of *S. juncea* and *S. reginae* to the growth medium containing excised *S. juncea* embryos was ascertained. Each treatment was replicated four times.

TABLE 1
Effect of seed coats of *S. juncea* and *S. reginae* on root length of excised *S. juncea* embryos.

Addition to culture medium	Mean root length of embryos (mm $\pm$ S.E.)
No addition (control)	21 $\pm$ 2,04
Seed coats of <i>S. juncea</i>	2 $\pm$ 0,25
Seed coats of <i>S. reginae</i>	3 $\pm$ 0,85

The results in Table 1 show that the presence of seed coats had a decided inhibitory effect on the growth of excised embryos. The seed coats of both species had a similar inhibitory effect.

In a second experiment a comparison was made between the inhibitory effects of seed coats of freshly-harvested and year-old seeds of *S. juncea* in the light and in the dark. For the light treatment, continuous lighting was supplied by eight cool white fluorescent tubes with a light intensity of 7 000 lux at flask level. Flasks of the dark treatment were placed in the same growth cabinet but were covered with a single layer of household aluminium foil to exclude light. All manipulations prior to placing embryos and seed coats into the flasks were performed as quickly as possible under normal laboratory lighting conditions. Treatments were randomised in a 3×2 factorial design with six replicates.

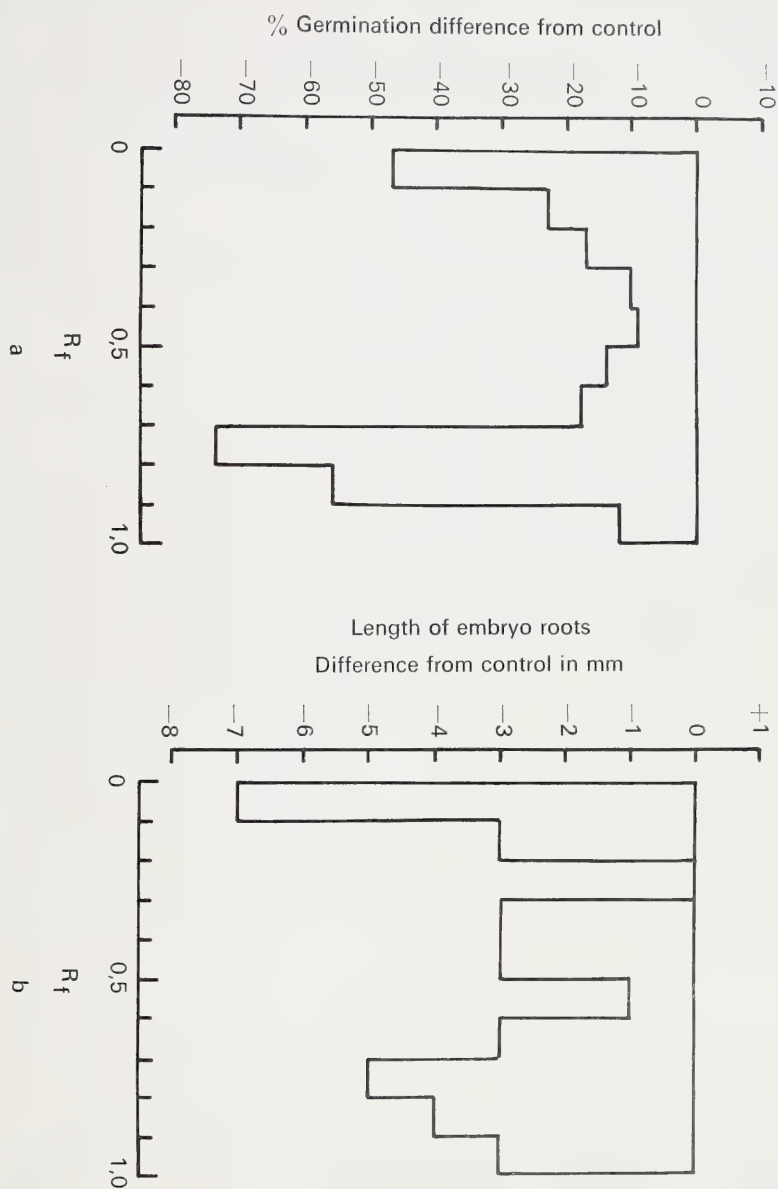


FIG. 2.

Inhibitory effect of aqueous extracts of *S. reginae* seeds.

(a) Effect on germination of lettuce seeds.

(b) Effect on root length of excised *S. juncea* embryos.

The results of this experiment (see Table 2) confirm the results of the first experiment concerning the inhibitory effect of seed coats on the growth of excised embryos. It can also be seen that the action of the inhibiting factor is as powerful in one-year-old seeds as it is in freshly harvested seeds.

TABLE 2

Effect of seed coats of different ages on the root length (in mm) of excised *S. juncea* embryos grown in light and darkness.

	Additions to growth medium of embryos			
	Control—no addition	Seed coats from freshly harvested seeds	Seed coats from year-old seeds	Mean
Light	29	6	7	14
Dark	35	6	4	15
Mean. . . .	32	6	6	

F value (seed coat effects): 80.56**

L S D (seed coat effects): 6 mm

The presence or absence of light did not influence the inhibiting action of seed coats. Growth of embryos in the absence of seed coats, however, was slightly better in the dark than in the light.

A further experiment was conducted on the effect of seed age on the inhibitory principle in seed coats. Seed coats of five-year-old *S. reginae* seeds were added to the growth medium of isolated embryos. A mean root length of 3 mm was recorded as compared to a root length of 23 mm in control treatments. The addition of seed coats of six-month-old seeds resulted in a root length of 2 mm.

In a fourth experiment, the effect of seed coats of germinated and ungerminated seeds of *S. reginae* on the growth of excised embryos was determined. The seed coats of germinated seeds were selected from seeds which had just started to germinate after incubation in petri dishes for two weeks. Seed coats of ungerminated seeds were obtained from seeds which had been allowed to imbibe water for only 15 hours. It was thought inadvisable to use ungerminated seeds which had been incubated for two weeks as there was no way of discerning which seeds were on the point of germination.

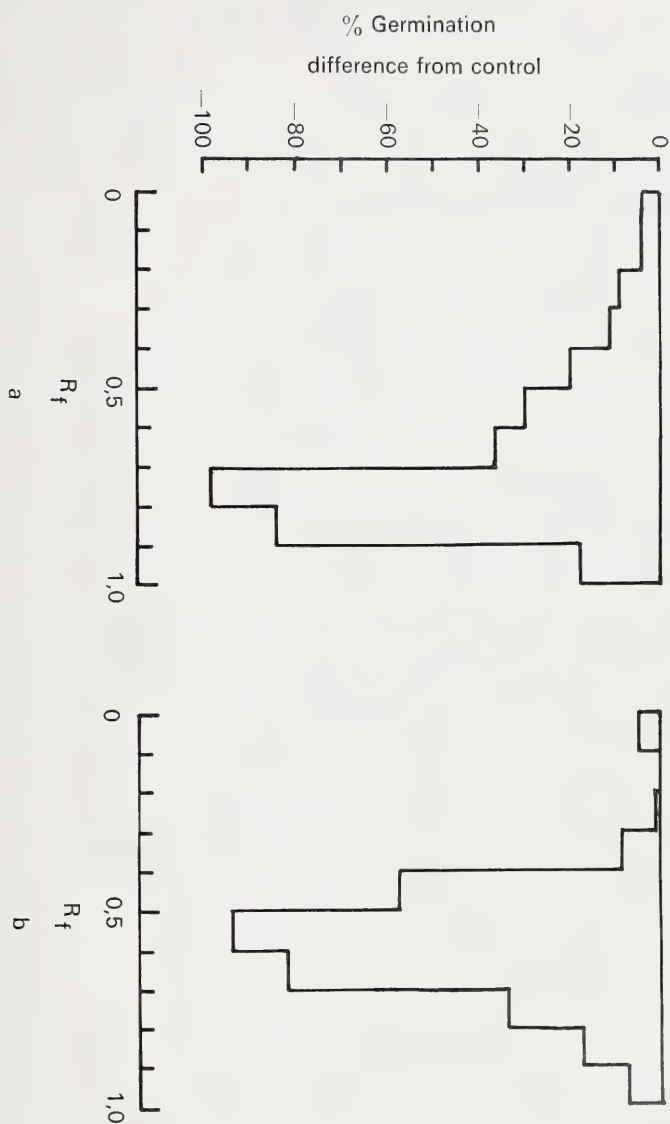


FIG. 3.
Effect of inhibitors on the germination of lettuce seed.
(a) Coumarin
(b) Absciscic acid.

Treatments were replicated six times. Three of the replicates containing seed coats from germinated seeds showed severe microbial contamination and were discarded. No sign of microbial growth was seen in any of the other flasks.

The results of this experiment are shown in Table 3 and embryos from the different treatments are pictured in Fig. 1.

TABLE 3

Effect of seed coats from germinated and ungerminated *S. reginae* seeds on the growth of *S. juncea* embryos.

Additions to culture medium	Mean embryo root length (mm)
Control—no addition.	27
Seed coats of ungerminated seeds	2
Seed coats of germinated seeds	17

F value: 73.03*

L S D: 6 mm

It can be seen that the effect of the inhibitor had been significantly reduced in the seed coats of germinated seeds. It is obvious, however, that the inhibitory effect had not been completely lost during germination as embryos growing in the presence of seed coats of germinated seeds showed significantly less growth than control embryos.

An experiment in which seed coats were added to the growth medium of actively growing embryos formed the basis of the bioassay reported in this paper. Treatments consisted of the addition of *S. reginae* seed coats to the growth medium of embryos which had been cultivated in isolation for three, six and nine days respectively. Treatments were also included where no seed coats were added at the commencement of incubation. Every treatment was replicated four times.

Twelve additional replicates of the control treatment were included in this experiment. After three, six and nine days of growth respectively, four control replicates were harvested to determine embryo root length at those stages. From these data it was possible to estimate the amount of embryo root growth that occurred after addition of seed coats. As asepsis had to be maintained, direct measurement of embryos at the time of adding seed coats could not be done. Increase in length after addition of seed coats was expressed as a percentage of the growth increase in untreated embryos over the same period.

TABLE 4

Root growth of excised *S. juncea* embryos after addition of *S. reginae* seed coats at different stages of growth.

Treatment	Final root length of embryo mm	Estimated increase in root length after addition of seed coats	
		mm	As % of control growth increase
Control—no seed coats added .	28	—	—
Seed coats added on first day .	4	4	14,0
Seed coats added after 3 days .	10	9	33,3
Seed coats added after 6 days .	9	4	17,4
Seed coats added after 9 days .	19	5	35,0

F value: 36,85** and L S D: 8 mm for final root lengths.

The results in Table 4 show that addition of seed coats to the growth medium significantly inhibited embryo root growth in all the treatments. The estimates of growth increase after addition of seed coats indicate that growth curtailment was severe.

Addition of seed coats to embryos which had already been growing for nine days resulted in their primary roots becoming swollen at their extremities. This can be interpreted as a greater inhibition of growth in length than in girth. It is interesting that coumarin is known to inhibit elongation of root cells while growth in width is apparently not affected (Avers and Goodwin, 1956 and Svensson, 1971). This phenomenon was not evident in any of the other treatments.

Extraction and chromatographic separation of the inhibiting principle

After having obtained evidence for the existence of an inhibitory factor in the seed coats of *Strelitzia* seeds, attempts were made to extract the inhibitor. The use of solvents such as methanol, acetone, ethyl acetate and chloroform gave results which were either negative or inconsistent. Consistent results were, however, obtained with aqueous extracts.

Figure 2A was compiled from the results obtained after extracts were made on two separate occasions. The lettuce germination biotest was replicated five times in the case of the first extraction and three times in the case of the second one. Similar results were obtained in both cases and the data was consequently pooled.

Two bands of inhibition were found on the chromatograms. Inhibition was found between R_f values 0,0 and 0,1 as well as between R_f 0,7 and 0,9. When viewed under ultraviolet light, the chromatograms also showed a light blue fluorescence in these regions.

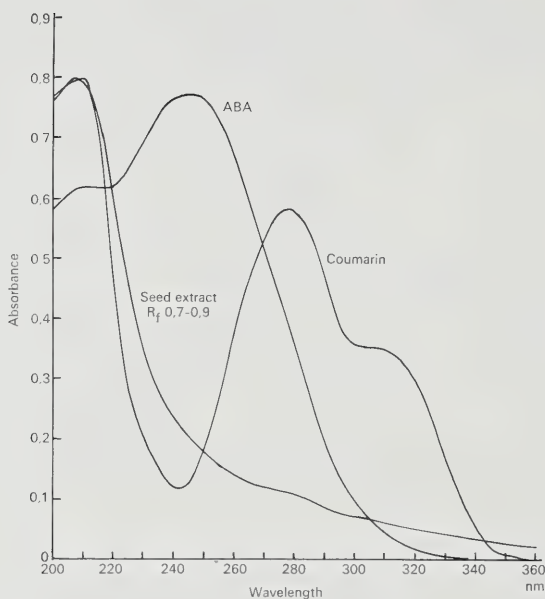


FIG. 4.

A comparison of the UV absorption spectra of abscisic acid (ABA), coumarin and an aqueous seed extract of *S. reginae*.

Peaks of inhibition were found at the same R_f values using the excised embryo biotest (Fig. 2B).

The results of tests measuring the biological activity of chromatograms of coumarin (Hopkin and Williams) and abscisic acid (Miles-Seravac) appear in Fig. 3.

Abscisic acid activity was found at R_f 0.4-0.7 which did not correspond to zones of inhibition found on chromatograms of seed extracts. In the case of coumarin, the activity at R_f values 0.7-0.9 coincided with the one zone of inhibition found on chromatograms of the seed extract.

Abscisic acid and coumarin were eluted from these zones on the chromatograms using distilled water, and their ultraviolet absorption spectra determined in a Pye Unicam SP 1800 spectrophotometer. The inhibitor in the seed extract partitioning at R_f 0.7-0.9 was also eluted in water and its absorption spectrum compared with those of ABA and coumarin. The absorption spectra are shown in Fig. 4.

Peak absorption was found at 245 nm in the case of ABA and at 277 nm in the case of coumarin. The absorption spectrum of the seed extract did not correspond to that of ABA or coumarin.

DISCUSSION

Working with dormant *Iris* seeds, Randolph and Cox (1943) found that excised embryos were capable of normal growth and development when placed on a suitable growth medium. When endosperm tissue was brought in intimate contact with these embryos, however, growth was markedly inhibited and progressed slowly in an atypical manner. Their conclusion that inhibitory substances were present in the endosperm of these seeds was confirmed when Kartaschoff (1958) was able to extract inhibitory substances from this tissue.

In the experiments reported in this paper, a similar approach was used and similar results were found. Excised embryos of *Strelitzia* were found to be capable of prompt and normal growth when placed in a suitable medium but growth was markedly inhibited on addition of seed coats. This suggests that the tissues surrounding the embryo contain an inhibitory substance which could possibly be related to the dormancy of the seed. The experiments have also shown that the effect of this inhibitor is not affected by the presence or absence of light and that it exists in the seed during extended periods of dry storage. Seeds of *S. reginae* do not retain their viability for very long (Van de Venter and Small, 1974) and a batch which had been stored for five years was found to have lost viability completely. In spite of this, the seed coats showed an inhibitory effect similar to that of fresh seeds. Seeds which, under natural conditions germinate before their viability is lost must, therefore, have some or other mechanism whereby they are able to overcome the effect of this inhibitor.

It was shown that seed coats of germinating seeds lose a great deal of their inhibitory effect. This does not necessarily imply a reduction in the absolute amount of inhibitor present. It is possible that the formation of a growth stimulator shifts the inhibitor/stimulator balance in favour of growth. The regulation of germination and growth by this type of interaction has been emphasized much in recent years by physiologists such as Amen (1968), Kefeli (1971) and Villiers (1972).

The presence of inhibitory fractions on chromatograms of aqueous extracts of *S. reginae* seeds provided further evidence for the existence of a germination inhibitor in the seed.

Altogether two zones of inhibition were found on the chromatograms. It is not known at present whether the inhibitory activity detected at R_f values 0,0–0,1 is caused by a "true" inhibitor or whether this zone merely represents a toxic effect. An aqueous extract contains many compounds such as proteins,

sugars and salts and the possibility that some of these compounds could affect germination adversely in the concentration occurring in the extract should be taken into account.

On the other hand, although inhibitory activity is evident in aqueous extracts of ground seeds, it was later found (unpublished results) that germination is not promoted when scarified seeds are subjected to an extensive leaching treatment. It is therefore possible that the greater portion of the inhibitor is bound *in vivo* to some other molecule in the seed. This bound form of the inhibitor might remain on the origin of the chromatogram while the free portion is able to descend to R_f 0.7–0.9. If this was the case the same inhibitor could be responsible for both zones of activity on the chromatogram.

There were reasons to suspect that the inhibitory effect of the seed coats could be ascribed to coumarin, a well-known naturally occurring germination inhibitor. The fact that in a certain case the presence of seed coats resulted in the swelling of root tips has already been mentioned as evidence of this. In germination experiments to be reported in a later paper, it was found that substances such as thiourea, cysteine and BAL (2,3-dimercapto-1-propanol) stimulated germination of dormant *S. reginae* seeds. These substances are known to reverse the inhibitory effect of coumarin (Nutile, 1945; Thimann and Bonner, 1949; Mayer and Evenari, 1952). It was also found that coumarin resulted in a zone of inhibitory activity at an R_f value of 0.7–0.9 on the chromatogram, corresponding to a similar zone of inhibition in the case of an aqueous seed extract. Absorption spectra of eluates have, however, failed to confirm any similarity between the inhibitor in the seed and coumarin. No evidence could be obtained that the inhibitor was ABA.

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